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PROGRESS REPORT NO. 5

APPLICATION OF MONOCLONAL ANTIBODIES AGAINST *ENTAMOEBA HISTOLYTICA* IN TROPICAL MEDICINE RESEARCH

A RESEARCH PROJECT

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SUBMITTED BY

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PROJECT PROFILE

Country	:	Thailand
Grant No.	:	936-5542-G-00-6029-00
Program	:	Program on Science and Technology Cooperation
Project Title	:	Application of monoclonal antibodies against *Entamoeba histolytica* in tropical medicine research
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Total Project Budget	:	U.S.$ 150,000
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OBJECTIVES

1. To type pathogenic *E. histolytica* by using mouse MABs.
2. To establish a sandwich ELISA technique for detection of amoebic antigen.

PROGRESS REPORT

1. Immunization of BALB/c mice with monoxenic culture of local strain of *E. histolytica*.
2. Isoenzyme typing of *E. histolytica*.
3. Isotyping of mouse MABs.
4. Western blot analysis of *E. histolytica* antigens recognized by MABs.
5. More typing of invasive and non-invasive *E. histolytica*.
6. Establishment of a sandwich ELISA technique for detection of *E. histolytica* antigen.
7. Establishment of axenic culture of *E. histolytica*.

1. IMMUNIZATION OF MICE WITH MONOXENIC CULTURE OF A LOCALLY-ISOLATED STRAIN OF E. HISTOLYTICA.

Immunization schedule of three BALB/c mice with monoxenic culture of HTH-56:MUTM was the same as reported in the Progress Report No. 2, except that the immunizing doses were equivalent to $1x10^5$, $2x10^5$, for the first two doses respectively and $4x10^5$ and $9x10^5$ for the last two doses. From three immunized mice, the IFA titers against homologous antigen coated slides were 1:5120, 1:1280 and 1:640 whereas the IFA titers against the HM1:IMSS strain were 1:2560, 1:1280 and 1:640 respectively. Spleens from these immunized mice will be fused with myeloma cells (P3-X63 Ag 8.653) when higher IFA titers are obtained.

2. ISOENZYME TYPING OF E. HISTOLYTICA.

Zymodeme typing of E. histolytica was carried out according to the technique described by Mr. P.G. Sargeaunt, London School of Hygiene and Tropical Medicine (1984). Of forty four isolates tested, 22 belonged to pathogenic zymodemes (20 zymodeme II, 1 zymodeme XIV and 1 zymodeme XIX), 18 to non-pathogenic zymodeme I, 2 to non-E. histolytica amoeba and the remaining two were untypable.

3. ISOTYPING OF MOUSE MABs.

Classes and subclasses of thirty eight MABs and 8

oligoclonal antibodies were determined by using mouse hybridoma isotyping kit from Calbiochem Immunochemicals. All belonged to subclass IgG, except one oligoclone (Eh6) which comprised of IgG1 and IgG2b.

4. WESTERN BLOT ANALYSIS OF E. HISTOLYTICA ANTIGENS RECOGNIZED BY MABs.

After fractionation of the trophozoites protein antigens of E. histolytica by 7.5-20% gradient sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the gel was electroblotted onto 0.45 um nitrocellulose membrane (Bio-Rad Laboratories) according to the method of Towbin et al. (1979) at a field strength of 300 mA for 18 hours at 26°C. The unreacted sites on the nitrocellulose strips after blotting were blocked by immersing the strips in PBS containing 2% gelatin 1% BSA, and 0.02% sodium azide (PBS-gel) at room temperature for two hour with gentle rocking. The blotted nitrocellulose strips were treated with immunized mouse serum, normal mouse serum, mouse Sp2/O supernatants and 21 monoclonal antibodies with gentle rocking for one and half hours at room temperature. The strips were then washed with rocking 5 times with PBS containing 0.05% Tween 20 and treated with ^{125}I-labeled rabbit anti-mouse IgG (1 x 10^5 cpm/ml in PBS/BSA from NEN) for one and half hours with agitation at room temperature. The strips were washed as previously described and dried. Autoradiography was done by exposing the strips to X-ray films (Kodak X-Omat RP) with

intensifying screen at -70°C.

Sera from immunized mice exhibited 12 precipitating bands with molecular weights ranging from 91 Kd to 18 Kd. Of 22 anti-E. histolytica MAB, 7 showed visible precipitation. Eh208C1 MAB reacted to 10 different amoebic proteins (61, 54, 46, 40, 38, 33, 27, 21, 18, 15 Kd) suggesting that these proteins have a common epitope. Eh6C12, Eh6D9, Eh6F9, Eh19C4, Eh35C1/4 and Eh35C5 reacted to 28 Kd amoebic proteins whereas the remaining 14 MABs did not show any visible precipitating bands with amoebic proteins. A MAB raised against P. falciparum was likewise non-reactive. Because of the absence of precipitating bands with anti-E. histolytica MABs, the test will be repeated again for verification.

5. MORE TYPING OF INVASIVE AND NON-INVASIVE E. HISTOLYTICA.

IFA screening test of cultured trophozoites of E. histolytica with 37 MAB were tested against 4 pathogenic zymodemes II (HTH-33:MUTM, HTH-34:MUTM, HTH-35:MUTM and HTH-56: MUTM), 2 non-pathogenic zymodemes I (HTH-37:MUTM and HTH-45:MUTM) and one non-E. histolytica amoeba (HTH-29:MUTM). These MABs were also tested against 2 reference pathogenic zymodeme II namely HM-1:IMSS and HK-9. The results are shown in Table 1. It was shown that Eh35C1 reacted to all pathogenic zymodemes but not to two non-pathogenic zymodemes

TABLE 1. IFA REACTIVITIES OF 37 MAB AGAINST CULTURED *E. HISTOLYTICA*

MAB	Strains	HTH-29	HTH-33	HTH-34	HTH-35	HTH-56	HTH-37	HTH-45	HM-1	HK-9
-	Zymodemes		II	II	II	II	I	I	II	II
Eh6C1		1+	1+	1+	2+	1+	2+	1+	3+	2+
Eh6C2		-	1+	1+	2+	1+	1+	1+	3+	2+
Eh6C2/8		ND	ND	ND	ND	-	ND	-	2+	2+
Eh6C12		-	2+	1+	1+	1+	1+	-	1+	1+
Eh6C13		-	2+	1+	1+	1+	1+	-	1+	1+
Eh6C14		1+	2+	2+	1+	1+	-	-	1+	2+
Eh6C18		-	2+	1+	1+	1+	1+	-	2+	2+
Eh6C19		-	2+	1+	1+	1+	1+	1+	2+	2+
Eh6C20		1+	1+	-	1	1+	1+	1+	1+	2+
Eh6C22		-	1+	1+	1+	1+	1+	-	2+	2+
Eh6C24		-	2+	-	1+	1+	1+	-	2+	2+
Eh6C26		-	2+	1+	1+	1+	1+	-	2+	2+
Eh19C1		-	-	-	-	-	-	-	1+	-
Eh19C4		1+	1+	1+	2+	2+	1+	-	3+	2+
Eh19C5		-	-	1+	1+	2+	1+	-	3+	2+
Eh19C6		-	-	-	-	1+	-	-	2+	1+
Eh19C7		-	1+	1+	1+	1+	1+	-	3+	2+
Eh19C8		-	1+	-	1+	1+	1+	-	3+	2+
Eh19C9		-	1+	1+	1+	1+	1+	-	2+	2+
Eh19C11		1+	2+	1+	1+	2+	1+	-	3+	2+
Eh19C13		1+	2+	1+	1+	2+	1+	-	3+	2+
Eh19C14		-	1+	1+	1+	2+	1+	-	3+	2+
Eh35C1		-	2+	2+	2+	3+	-	-	3+	2+
Eh35C1/4		ND	ND	ND	ND	2+	ND	-	3+	ND
Eh35C3		1+	2+	1+	2+	3+	-	-	3+	2+
Eh35C4		1+	1+	1+	1+	2+	-	-	3+	2+
Eh35C5		-	-	-	-	-	-	-	3+	1+
Eh16C1		-	-	-	-	1+	-	-	2+	2+
Eh16C2		-	-	-	-	-	-	-	1+	1+
Eh16C3		-	-	-	-	ND	-	-	1+	1+
Eh16C7		-	-	-	-	-	-	-	2+	2+
Eh16C8		-	-	-	-	1+	-	-	2+	2+
Eh106C1		-	ND	-	ND	2+	ND	-	3+	3+
Eh208C1		1+	ND	-	ND	3+	ND	4+	3+	3+
Eh208C2		-	ND	1+	-	3+	ND	1+	3+	3+
Eh208C3		-	ND	1+	-	3+	ND	1+	3+	3+
Eh208C6		-	ND	-	-	3+	ND	1+	3+	3+

NOTE : MAB Eh35C1 REACTS TO ALL FOUR PATHOGENIC ZYMODEMES BUT NOT TO TWO NON-PATHOGENIC ZYMODEMES.

6. AN ATTEMPT TO ESTABLISH A SANDWICH ELISA TECHNIQUE FOR DETECTION OF *E. HISTOLYTICA* ANTIGEN.

Culture supernate of the monoclone Eh208 was collected, concentrated by precipitation with 50% $(NH_4)_2SO_4$ precipitation, extensively dialysed against physiological saline at 4°C, and sterilized through a 0.45μ Millipore membrane. A block titration was carried out. Wells of a 96-well plate (Immulon, Dynatech) was coated with 100 ul of Eh208 MAB at 100, 50, 25 and 10 ug/ml in a carbonate-bicarbonate buffer pH 9.6, and kept at 37°C for 1 hour and at 4°C overnight. The plate was washed with PBS and blocked for 1 hour with 100 μl of PBS-T-1%BSA (phosphate buffered saline with 0.05% Tween-20 and 1% bovine serum albumin). After washing, 100 ul of a serial five-fold dilutions in PBS-T-0.25%BSA (PBS-T with 0.25% BSA) of the physiological saline extract of the HM-1 strain of *E. histolytica* with the initial concentration of 500 μg/ml was added to the plate, followed by incubation at 37°C for 1 hour. The plate was washed, and blocked with 100 μl of PBS-T containing 1% bovine serum albumin (Sigma) for one hour at 37°C. After washing, the plate was washed and blocked for one hour with PBS-T 1% BSA and incubated further for one hour. Thereafter, the plate was washed and 100 μl of 1:100 dilution of rabbit anti-*E. histolytica* added. After incubation for one hour at 37°C, the plate was washed and 100 μl of 1:300 dilution of swine anti-rabbit immunoglobulin (Dakopatts) added, followed by incubation at 37°C for one hour. The plate was again washed and a substrate O-pheynilinediamine (OPD) added, followed by incubation at 37°C for another 30 min. The reaction was

stopped with 1N H_2SO_4 and the result read with a Multiskan ELISA reader (Titertrek) at 492 nm. Only one experiment has been performed and the result (table 2) shows that plate coating with 25 µg/ml of Eh208 is optimum, and the assay can detect as small as 0.03 ug/ml of E. histolytica antigen. This assay at present is time-consuming and has to be modified and then applied for detection of E. histolytica antigen in clinical specimens.

TABLE 2. BLOCK TITRATION OF Eh208 MAB FOR USE IN ELISA FOR DETECTION OF E. HISTOLYTICA ANTIGEN

Eh208 MAB (µg/ml)	ANTIGEN CONCENTRATION (µG/ML)							PBS CONTROL
	500	100	20	4	0.8	0.16	0.033	
100	0.629	0.629	0.523	0.407	0.460	0.396	0.406	-0.012
50	0.719	0.693	0.846	0.590	0.483	0.482	0.468	-0.004
25	0.785	0.735	0.627	0.593	0.577	0.554	0.528	0.004
10	0.687	0.678	0.627	0.588	0.601	0.566	0.558	0

7. ESTABLISHMENT OF AXENIC CULTURE OF E. HISTOLYTICA.

Attempts were made to grow E. histolytica from liver pus aspirated form 7 Thai patients with proven amoebic liver abscess in TYI-S33 medium containing 15% heat-inactivated normal horse serum. Continuous monoxenic culture with a crithidia strain has been established only with the strain from Mr. Vasana Sriburom (HTH-56:MUTM) (Progress Report No. 4). After removal of crithridia, we have succeeded in axenizing this local strain of amoebae (Fig. 1).

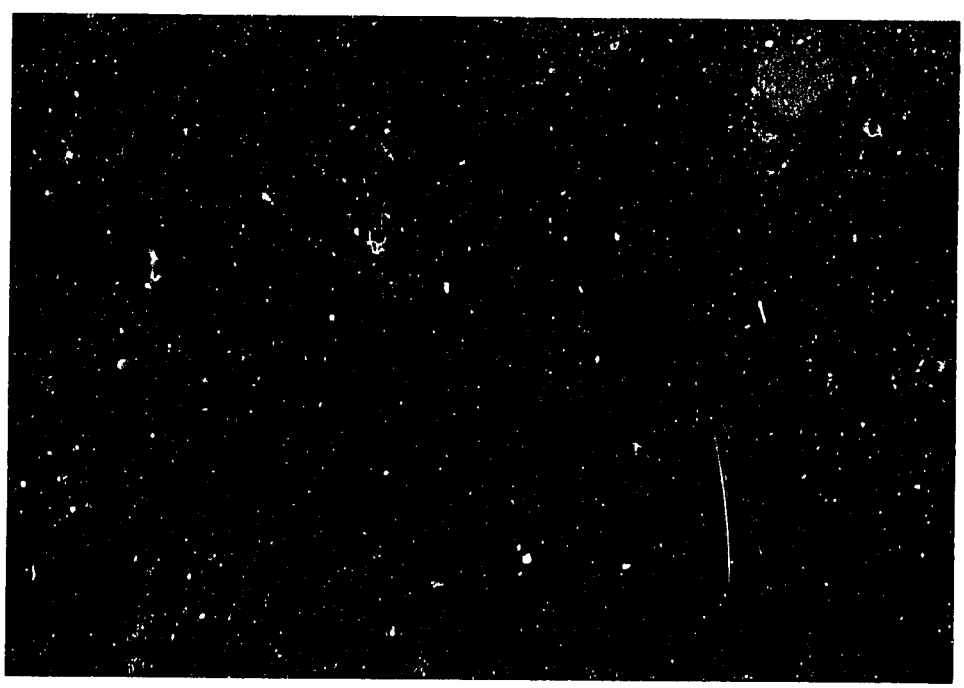

Fig. 1 Axenic culture of HTH-56:MUTM *in vitro*